STATEMENT OF WORK

A VALIDATION STUDY FOR *IN VITRO* BASAL CYTOTOXICITY TESTING

BALB/c 3T3 Neutral Red Uptake Cytotoxicity Assay
and
Normal Human Keratinocyte Neutral Red Uptake Cytotoxicity Assay

June 21, 2002

Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

Based on Standard Operating Procedure Recommendations from an International Workshop Organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

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1.0 PROJECT OBJECTIVES AND GENERAL REQUIREMENTS

1.1 Project Objectives

This Statement of Work outlines and supports the procedures for performing two *in vitro* basal cytotoxicity assays (the BALB/c 3T3 Neutral Red Uptake [NRU] assay and the Normal Human Keratinocyte [NHK] Neutral Red Uptake [NRU] assay) for analysis of test chemicals for a multi-laboratory *in vitro* Validation Study. These *in vitro* assays, recommended in *Guidance Document On Using In Vitro Data To Estimate In Vivo Starting Doses For Acute Toxicity* (ICCVAM, 2001a) use mammalian cell culture techniques to assess the basal cytotoxicity of chemicals.

A primary goal of this Validation Study is to evaluate the usefulness and effectiveness of *in vitro* basal cytotoxicity assays for reducing and refining animal use for acute oral toxicity determinations of chemicals by predicting starting doses for *in vivo* rodent acute lethality assays. Participants at an international workshop (ICCVAM, 2001b) suggested that a validation study for *in vitro* assays is needed to continue the development of alternative tests as replacements for animal testing. This is the first step to further standardization and evaluation of two test methods that may be used in conjunction with other methods as components of a test battery which may eventually replace the rodent acute oral toxicity tests.

Data will be used to:

1) Develop standardized *in vitro* basal cytotoxicity protocols with sufficient detail and instruction for distribution to other laboratories (e.g., Federal regulatory agencies) for their immediate use,
2) Evaluate the intra- and inter-laboratory reproducibility of the assays (i.e., to access test reproducibility and optimize to further enhance reproducibility),
3) Determine the reduction in the number of animals that would be used and/or killed in lethality assays compared with the conventional method of predicting starting doses, and
4) Assess the relevance of the two standardized *in vitro* cytotoxicity assays for estimating rodent oral LD50 values across the six Globally Harmonised System (GHS; OECD, 2001) categories of acute oral toxicity and estimating human lethal concentrations.

This study will test the hypothesis of the Registry of Cytotoxicity (RC) prediction model (Halle, 1998) by comparing the NRU regressions that are developed from the two assays to the RC regression. The hypothesis is that the two NRU assays will provide the same regression as the RC (i.e., comparison of IC_{50} data vs. LD_{50} data).

The proposed Validation Study will provide the means to determine IC_{20}, IC_{50}, and IC_{80} values for a test set of 72 chemicals with varying degrees of toxicity. This set of chemicals was selected separate and prior to this Statement of Work by the Study Management Team. The basis for selection of this test set is discussed in the Study Design document prepared by the Study Management Team.

1.2 Response to the Statement of Work

The proposals submitted in response to the Statement of Work to the designated contacts shall include:

a) A timetable for project milestones
b) A cost estimate for performing all testing (both assays) in all phases of the Validation Study.
c) Cost estimates for repeating Phases Ia, Ib, and II as options, if necessary (see **Sections 4.2.2, 4.2.4, and 4.3.2**).
d) Cost for a third replicate of Phase III testing as an option, if necessary
e) Cost of software for data analysis (e.g., GraphPad PRISM® 3.0) not to exceed $500.

1.2.1 *General Capabilities*

The contracted laboratories (Testing Facilities) shall be capable of performing the following:

a) The Testing Facilities shall prepare Standard Operating Procedures (SOPs) for the 3T3 NRU assay and the NHK NRU assay (see **Section 1.4** – Definitions - SOPs)
b) The Testing Facilities shall perform the 3T3 NRU assay and the NHK NRU assay (under aseptic *in vitro* laboratory conditions) for the three phase Validation Study as identified in **Section 4.0**.
c) The Testing Facilities shall provide IC_{20}, IC_{50}, and IC_{80} values for each tested chemical and other information addressed in this document (e.g., phase reports) to the Study Management Team through the designated contacts (**Section 2.2**).
d) Testing Facilities that are compliant with Good Laboratory Practices (GLP) shall perform all aspects of the Validation Study in accordance with GLPs.
e) Testing Facilities that are not GLP-compliant shall perform all aspects of the Validation Study "in the spirit" of GLP which is defined in **Section 1.4** and addressed throughout this Statement of Work.
f) All Testing Facilities shall adhere to this Statement of Work throughout the Validation Study.

1.3 Guidelines

The Management Team and/or its representatives may inspect and audit the Testing Facilities used for this study to ensure that the Study Management Team's minimum requirements and guidelines are being followed. The contractor shall notify the Study Management Team of any changes in Key Personnel (identified in Section 3.1.1)

1.4 Definitions

Blinded/Coded Chemicals: Test chemicals supplied to the Testing Facilities that are coded (by an NIEHS/NTP-designated contractor) such that the Testing Facilities do not know the identity of the chemicals. Only the Project Officer, Management Team, and contractor know the contents of each test chemical vessel. The test chemicals will be purchased, aliquoted, coded, and distributed by a contractor under the guidance of the NIEHS Project Officer and the Management Team.

Good Laboratory Practices (GLPs): Regulations governing the conduct, procedures, and operations of toxicology laboratories; regulations to assure the quality and integrity of the data and to address such matters as organization and personnel, facilities, equipment, facility operations, test and control articles, and Validation Study protocol (Statement of Work) and conduct (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160).

IC_X: Inhibitory concentration estimated to affect endpoint in question by X % (IC_{20} = 20 % affected; IC_{50} = 50 % affected; IC_{80} = 80 % affected).

Lead Laboratory (Protocols): A designated laboratory (identified by the Study Management Team and different from the lead laboratory for data analysis) with experience in each cytotoxicity method. The laboratory will assist the Study Management Team with troubleshooting laboratory challenges; the lead laboratory shall develop a study protocol from the Statement of Work and the Test Method Protocols that shall be used by all laboratories in the Validation Study.

Lead Laboratory (Data Analysis): A designated laboratory (identified by the Study Management Team and different from the lead laboratory for protocols) with experience in data analysis specific to the software that will be used in the study; The laboratory will assist the Study Management Team with troubleshooting data analysis challenges.

Replicate: An independent test run on different days (e.g., duplicate 96-well plates for a particular test chemical, each plate a replicate assay); replicate wells within the 96-well plate (e.g., six wells of one test chemical concentration equals six replicate wells).

Spirit of GLP: Laboratories that are non GLP-compliant shall adhere to GLP principles and other method parameters as put forth in this Statement of Work and the Test Method Protocols (provided by NIEHS/NICEATM); documentation and accountability shall be equal to GLP requirements; laboratories must make assurances that they are equal in performance criteria and that there is parity amongst the laboratories.

Standard Operating Procedures (SOPs): Written documents that describe, in great detail, the routine procedures to be followed for a specific operation, analysis, or action; consistent use of an approved SOP ensures conformance with organizational practices, reduced work effort, reduction in error occurrences, and improved data comparability, credibility, and defensibility; SOPs also serve as resources for training and for ready reference and documentation of proper procedures; each Testing Facility involved in the Validation Study shall draft SOPs specifically for its laboratories based on: protocols supplied by commercial sources specifically for cell culture products and cell lines; this Statement of Work and the Test Method Protocols provided by NIEHS/NICEATM, and the study protocol developed by the lead laboratory.

Statement of Work: A description of testing required for the *in vitro* Validation Study; defines all phases of the Validation Study and the purpose of the procedures; provides the details of the experimental design, data acquisition, data analysis, and preparation of reports; supports Test Method Protocols (equivalent to GLP protocols) and acts as a study plan.

Study Protocol: A description of the objectives and all methods for the conduct of the study (i.e., same as "protocol" according to GLP guidelines, 40 CFR 792, at http://www.ovpr.uga.edu/qau/tscatoc.html. The Study Protocol shall be developed from the Test Method Protocols for NHK and 3T3 NRU assays, which accompany this Statement of Work. The Study Protocol shall contain information such as the title and purpose of the study, name and address of the sponsor, the name and address of the testing facility at which the study is being conducted, proposed experimental start and termination dates, and other items specified in 40 CFR 792.

Test Method Protocols: Specific and detailed guides for performing the 3T3 NRU and NHK NRU cytotoxicity assays; adapted by NICEATM from protocols included in ICCVAM (2001a); equivalent to GLP protocols; protocols shall be incorporated into the SOPs specific to each Test Facility in the Validation Study.

2.0 ORGANIZATION

2.1 Validation Study Sponsors

- National Institute of Environmental Health Sciences (NIEHS)
- The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
- U.S. Environmental Protection Agency (U.S. EPA)
- The European Centre for the Validation of Alternative Methods (ECVAM).

2.2 Management Team

2.2.1 *Study Management Team*

2.2.1.1 ***NIEHS/NICEATM***

Dr. William S. Stokes (NICEATM/NIEHS) – Co-chair – Study Management Team
Dr. Judy Strickland (NICEATM/ILS) – Project Coordinator
Mr. Michael Paris (NICEATM/ILS) – Assistant Project Coordinator
Dr. Ray Tice (NICEATM/ILS) – Technical Advisor

NICEATM
79 T.W. Alexander Drive
Bldg. 4401, MD-EC-17
3rd Floor, Room 3126
P.O. Box 12233
Research Triangle Park, NC 27709

2.2.1.2 ***ECVAM***

Professor Michael Balls – Co-chair – Study Management Team
Dr. Silvia Casati
Dr. Andrew Worth

European Commission
Joint Research Centre
Institute for Health and Consumer Protection
Management Support Unit - TP 202
I-21020 Ispra (VA) - Italy

2.2.2 *Project Management and Chemical Distribution Team*

Ms. Molly Vallant (NIEHS) – NIEHS Project Officer for BioReliance, Inc.
Dr. Martin L. Wenk (BioReliance, Inc., Rockville, MD) – Principal Investigator/Chemical Distribution

2.2.3 *Contract Management*
Ms. Jackie Osgood (NIEHS) – Contracting Officer
Mr. Don Gula (NIEHS) – Contracting Officer

3.0 TESTING FACILITY AND KEY PERSONNEL

3.1 Testing Facility
The Testing Facility shall have competence in performing *in vitro* cytotoxicity assays under aseptic laboratory conditions and shall provide competent personnel, adequate facilities, equipment, supplies, proper health and safety guidelines, and satisfactory quality assurance procedures.

3.1.1 *Personnel*

3.1.1.1 ***Facility Management***
The facility management is responsible for establishing scientific guidelines and procedures, training and supervision of professional and technical staff, and evaluation of results and performance within their discipline area relative to the Study Management Team requirements. The manager must maintain records of the qualifications, training and experience, and a job description for each professional and technical individual involved in the Validation Study.

3.1.1.2 ***Study Director***
A scientist or other professional of appropriate education, training, and experience in *in vitro* cytotoxicity assay performance, or combination thereof, shall be the Study Director. The Study Director has the overall responsibility for the technical conduct of the Validation Study (e.g., GLP adherence or implementation of spirit of GLP) at the Testing Facility and shall be responsible for determining test acceptance. The Study Director shall be responsible for providing SOPs for the Validation Study and incorporating pertinent information obtained from the Statement of Work and the Test Method Protocols. Other duties include the interpretation and analysis of data, documentation of all Validation Study aspects (including maintenance of a Study Workbook), and production of all draft and final written Validation Study reports.

3.1.1.3 ***Quality Assurance (QA) Director***
For Testing Facilities that are GLP-compliant, the Quality Assurance Director shall **monitor** the Validation Study to assure conformance with GLP requirements for all aspects of the Validation Study (i.e., facilities, equipment, personnel, methods, practices, records, controls, transference of data into software, SOPs). The Quality Assurance Director or unit can be any person or organizational element, except the Study Director, designated by Testing Facility management to perform the duties relating to quality assurance of the studies. The Quality Assurance duties are not a substitute for the Study Director duties.

For Testing Facilities performing the Validation Study in the spirit of GLP, management shall appoint an individual to assure that all records, documents, raw data, reports, and specimens are available to the Management Team through the designated contacts if an inspection is requested.

3.1.1.4 *Scientific Advisor(s)*

Scientists or other professionals of appropriate education, training, and experience in *in vitro* laboratory methods and techniques who provide scientific guidance to the Study Director and other laboratory personnel.

3.1.1.5 *Laboratory Technician(s)*

In vitro cytotoxicity assays require personnel trained in sterile tissue culture techniques and general laboratory procedures. At least two individuals must be capable of performing the *in vitro* assays for the Validation Study. Performance of the assays requires a relatively moderate degree of technical capability and a high degree of technical accuracy. Each individual engaged in the conduct of or responsible for the supervision of a Validation Study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties. The individuals in a GLP-compliant laboratory must be trained in GLP requirements and technical ability must be documented as per GLP requirements. Non GLP-compliant laboratory personnel must be able to perform all aspects of the Validation Study in the spirit of GLP.

3.1.1.6 *Safety Officer*

A designated Safety Officer (someone not involved in the actual conduct of the Validation Study) at each participating laboratory will receive the blinded (coded) test chemicals from an NIEHS/NTP-designated contractor (BioReliance) and shall transfer the test chemicals to the Study Director without revealing the contents of the test chemical containers. A sealed health and safety information package will accompany the test chemicals and the Safety Officer shall retain the package until the completion of the Validation Study. At the end of the Validation Study, the Safety Officer shall return the unopened package to the contractor (BioReliance). If any Test Facility personnel should open the package at any time during the Validation Study, the Safety Officer shall notify the Management Team through the designated contacts.

3.1.2 *Facilities, Equipment, and Supplies*

3.1.2.1 *Cell Culture Laboratory*

Each Testing Facility must provide a designated cell culture laboratory to ensure that *in vitro* cytotoxicity assays can be performed under clean and proper aseptic conditions. The laboratory must be located such that there is minimal through traffic to reduce possible disturbances that may compromise the cell culture assays. Room temperature of the laboratory must be easily regulated, monitored, and documented. Access to the Validation Study assays and test chemicals shall be restricted to appropriate personnel as determined by facility management.

3.1.2.2 *Equipment*

Each Testing Facility must provide at a minimum the following equipment:

a) Laminar flow hood (biohazard type and restricted to cell culture assays)
b) Cell culture incubators
  - $37^oC \pm 1^oC$, 5 % ± 1 % CO_2, 90 ± 5 % humidified
c) Low-speed centrifuge
d) Water bath (37^oC)
e) Inverse phase microscope
f) Pippettors (multichannel pipettor, micropipettors, multichannel pipette units)

g) Spectrophotometric plate reader (equipped with a 540 nm ± 10 nm filter)
h) Computer (for data transformation and analysis)
i) Liquid nitrogen freezer (for storage of cryopreserved cells)
j) Refrigerator (4°C)
k) Freezers (-20°C and -70°C to -80°C)
l) Autoclave (for instruments and for biohazardous waste materials)
m) Balance
n) pH meter
o) Cell counting system (e.g., hemocytometer, Coulter counter)
p) General cell culture laboratory equipment (e.g., glassware, filtration systems, cell culture plasticware, etc.)
q) pH paper (wide and narrow range)

All equipment maintenance and calibration shall be routinely performed and documented as per GLP guidelines (or spirit of GLP for non GLP-compliant laboratories) and Testing Facility procedures. Additional detail is provided in **Section 10.3** and Addendum IV.

3.1.2.3 *Supplies*

a) General cell culture materials and supplies are needed and are specifically described in the provided Test Method Protocols and in the *Guidance Document* (ICCVAM, 2001a). All cell culture reagents must be labeled so as to indicate source, identity, concentration, stability, preparation and expiration dates, and storage conditions.
b) BALB/c 3T3 mouse cells, clone 31
    - Cryopreserved (5 vials, same lot)
    - CCL-163, *LGC Reference Materials*, Customer Service, Queens Road, Teddington, Middlesex, TW110LY, UK (http://www.lgc.co.uk/atcc/)
    - CCL-163, *American Type Culture Collection (ATCC)*, Manassas, VA, USA (http://www.atcc.org/)
c) Normal Human Epidermal Keratinocytes (NHK)
    - Cryopreserved (20 vials, same lot, first passage)
    - Non-transformed cells; from cryopreserved primary cells (**Clonetics #CC-2507** [pooled neo-natal keratinocytes]**)**
    - *Clonetics/BioWhittaker* [BioWhittaker, 8830 Biggs Ford Road, Walkersville, MD 21793-0127 (http://www.cambrex.com/subsidiaries/s%2Dbw%5Finc/s%2Dbiowhittaker%2Dinc%2Dcontact2.htm)
    - *BioWhittaker Europe* [BioWhittaker Europe, S.P.R.L. Parc Industriel de Petit Rechain, B-4800 Verviers, BELGIUM] (http://www.biowhittaker.be/index.htm)

3.1.3 *Health and Safety*

Each Testing Facility shall conform to all local, state, and federal statutes in effect at the time of this Validation Study. The designated Safety Officer shall be the point of contact for health and safety issues.

3.1.4 *Quality Assurance*

3.1.4.1 ***GLP-Compliant Laboratories***

GLP-compliant laboratories shall conduct this Validation Study in compliance with Good Laboratory Practice (GLP) Standards (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160). The appropriate QA unit (as per GLPs) shall review the protocol and audit the in-life phase, laboratory notebooks, and final report data.

The Final Reports for all phases of the Validation Study shall be audited by the Quality Assurance unit of the Testing Facility for GLP compliance and a QA Statement shall be provided by the Testing Facility. Each Final Report shall identify: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study.

3.1.4.2 ***Non GLP-Compliant Laboratories***

Non GLP-compliant laboratories shall use GLP standards referenced in the ECVAM Workshop 37 Report (Cooper-Hannan, 1999) and the OECD Principles of GLP (OECD, 1998) as guidelines for conducting the Validation Study in the spirit of GLP.

At a minimum, the following laboratory parameters and equipment must be routinely documented (e.g., log books; see Addendum IV). The documents shall be archived such that they can be available to the Study Management Team through the designated contacts upon request.

Daily Documentation (value, time, and date)

- Laboratory: room temperature
- Incubators: temperature, %CO_2, %humidity
- Water bath: temperature
- Refrigerators and freezers: temperature
- Cell cultures: visual observations (see Test Method Protocols)

Per Use Documentation (value, time, and date)

- Cryogenic storage unit: amount of liquid N_2 in container; when liquid N_2 added
- Balance: standard weight used to calibrate
- pH meter: values for standards used to determine slope
- Cell counter: standard used
- Media: identification of all media and components used

Periodic Documentation

- Media and components: date of receipt; lot numbers; expiration dates
- 3T3 and NHK cells: date of receipt; lot number; storage conditions
- Plastic tissue-culture ware (sterile, disposable): stock and lot numbers
- Computer software: identification and description
- Calibration of Instruments: SOPs for laboratory equipment
  - Incubators
  - Laminar flow hoods

Autoclaves
Micropipettors
Balances
pH meters
Cell counters
Refrigerators
Freezers
Water baths
Spectrophotometer plate readers

A statement from the Testing Facility shall be included with each Final Report and shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study and provide assurance that all testing was done in the spirit of GLP.

4.0 TEST PHASES AND SCHEDULE

See Addendum VI for Gantt Chart of study timelines and deliverables.

4.1 Study Timeline and Deliverables

TASK	WEEK	ESTIMATED DATE
Statement of Work issued by NIEHS to the Testing Facility	0	March 29, 2002
Response /Proposal received from the Testing Facility	6	May 10, 2002
Award of Contracts	13	June 28, 2002
Submission of Study Protocol, CVs of Key Personnel and SOPs	15	July 12, 2002
Start Testing – Phase I (Phase Ia)	18	July 29, 2002
End Phase Ia	22	August 26, 2002
Begin Phase Ib	26	September 26, 2002
End Phase Ib	31	October 29, 2002
Begin Phase II	36	December 2, 2002
End Phase II	46	February 10, 2003
Begin Phase III	52	March 26, 2003
Final Report (Phase III) to SMT	89	December 9, 2003

4.1.1 Deliverables

	ESTIMATED DUE DATES			
REPORTS	**PHASE 1a**	**PHASE Ib**	**PHASE II**	**PHASE III**
Biweekly	*	*	*	*
Draft	Week 24 Sept. 9, 2002	Week 33 Nov. 11, 2002	Week 48 Feb. 25, 2003	Week 82 Oct. 24, 2003
Final	Week 33 Nov. 11, 2002	Week 42 Jan. 13, 2003	Week 57 April 28, 2003	Week 89 Dec.9, 2003
Study Workbook (Draft)	Week 24 Sept. 9, 2002	Week 33 Nov. 11, 2002	Week 48 Feb. 25, 2003	Week 82 Oct. 24, 2003
Study Workbook (Final)	Week 33 Nov. 11, 2002	Week 42 Jan. 13, 2003	Week 57 April 28, 2003	Week 89 Dec.9, 2003

* Biweekly reports shall begin at the time of implementation of the contracts and continue until the final report is submitted.

4.2 Phase I

Phase I will be the training phase for laboratory personnel. This phase includes developing a positive control database (Phase Ia) and testing three unknown chemicals (Phase Ib). SOPs for the two NRU cytotoxicity assays shall be developed by the appropriate laboratory personnel prior to implementation of test procedures (See **Section 1.4** – Definitions – SOPs). They will be submitted along with the signed protocols to the designated contacts before initiation of Phase I.

4.2.1 *Study Procedures*

4.2.1.1 ***Phase Ia: Positive Control Database***

An historical database of $\mathbf{IC_{50}}$ values for the positive control chemical (Sodium Lauryl Sulfate [SLS]) will be established and maintained for each NRU assay by performing 10 concentration-response assays (10 microtiter plates, one plate per assay) on both cell types. A range finder experiment will be performed before initiating the 10 concentration-response assays (**Section 9.3**). The Test Facility personnel shall prepare and test eight concentrations (per microtiter plate) of the positive control chemical by diluting the stock solution with a constant factor for the range finder experiment (e.g., log dilutions [1:10, 1:00, 1:1000, etc.]). For the definitive concentration-response assays, the Study Director shall use a $\sqrt[6]{10} = 1.47$ dilution scheme centered on the IC_{50} identified in the range-finding assay.

Once a range has been determined that satisfies the criteria in **Section 11.2**, then the Test Facility shall perform two tests per day (each assay) on five different days. Control limits for the positive control chemical shall be established and a draft report (including range finding data) shall be provided to the designated contacts. After evaluation of the data, the Management Team will decide when to advance to the next phase of the Validation Study.

The 95 % confidence interval (CI) of the IC_{50} of SLS will be established and defined as an acceptance criterion for test sensitivity for the 3T3 NRU and NHK

NRU assays. The confidence intervals shall be calculated using the average of the individual IC_{50} values from each positive control assay performed. An example of an historical mean IC_{50} of SLS in mammalian cultures is **93 µg /ml** and the 95 % CI is **70 - 116 µg /ml** (Spielmann et. al., 1991). An example of an historical mean IC_{50} of SLS in NHK cultures is **4.4 µg/ml ± 0.97 µg/ml [**two standard deviations] (Triglia, 1989).

The following 96-well plate configuration will be used for the positive control assays.

Figure 1. 96-Well Plate Configuration for Positive Control Assays (Phase Ia)

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
C	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
D	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
E	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
F	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
G	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

VC = untreated VEHICLE CONTROL (mean viability set to 100 %)
$C_1 – C_8$ = POSITIVE CONTROL (SLS) at eight concentrations (C1 = highest, C8 = lowest)
b = BLANKS (contain **no** cells)

4.2.1.2 ***<u>Reporting Positive Control Data (Phase Ia)</u>***
<u>Biweekly Reports</u>: Each testing facility will provide a biweekly progress report to the designated contacts. These reports will be provided in electronic format (i.e., email with attachments) and will include raw and interim data as the study progresses. The Management Team will in turn provide a weekly progress report addressing the Validation Study as a whole to all of the Testing Facilities.

<u>Draft Report</u>: At the conclusion of Phase Ia, a draft report of the positive control data shall be provided by the Study Director to the designated contacts. The draft report (entitled: *In Vitro Validation Study – Phase Ia: Development of a Positive Control Database in Rodent and Human Cell Systems*) shall include everything noted in Addendum I (Draft Report – Phase Ia). If the Phase Ia data does not meet test acceptance criteria, then the Management Team (through the designated contacts) will work with the Test Facility and lead laboratory to identify problems and make corrections as needed. Once unresolved issues have been resolved, the

Validation Study will proceed. The Validation Study will advance to Phase Ib once all participating laboratories have submitted acceptable draft reports. The draft report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the unaudited Study Workbook pages may be submitted as attachments in hard copy format.

Final Report: Once the draft report provides data that meets test acceptance criteria, then the Project Coordinator shall inform the Study Director to prepare a Quality Assurance audited final report for Phase Ia. The final report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the audited Study Workbook pages may be submitted in hard copy format as an attachment to the report. The final report will not need to be completed to continue to Phase Ib. (See Validation Study timelines in **Section 4.1** and Report submission timelines in **Section 4.5**.)

4.2.2 *Criteria for Advancing to Phase Ib*

If there is excessive variation of ICx data within or among laboratories involved in the Validation Study, the lead laboratory for each method shall assist the Management Team (through the designated contacts) to determine the cause and recommend appropriate actions needed to reduce the variation. The Statement of Work, Test Method Protocols, and SOPs shall be revised if necessary, and testing repeated until acceptable proficiency is achieved. The Management Team will decide when all laboratories will advance to the next phase of the Validation Study. A teleconference shall be held with all of the appropriate participants of the Validation Study and the Management Team will relate information concerning the advancement of the Validation Study.

4.2.3 *Study Procedures*

4.2.3.1. ***Phase Ib: Chemical Testing***

Three blinded/coded chemicals with varying cytotoxicity (high, medium, and low) will be tested in both NRU assays. Eight concentrations of each chemical will be tested in a 96-well plate (six wells per concentration) with at least four replicates per concentration required for data analysis (**Section 12.0**). Only one test chemical will be tested on each plate. The assay setup will follow the 96-well (microtiter) plate configuration in Figure 2. A range finder experiment will be performed before initiating concentration-response assays (**Section 9.3**). After the range finding assay is completed, the concentration-response experiment shall be performed three times on three different days for each assay and each chemical. Laboratories will **calculate IC_{20}, IC_{50}, and IC_{80} values in μg/ml,** calculate confidence limits for each value, and report this and all raw data to the Management Team through the designated contacts.

Figure 2. Plate Configuration for 3T3 NRU and NHK NRU Assays (Phase Ib)

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
C	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
D	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
E	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
F	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
G	b	VC	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

VC = untreated VEHICLE CONTROL (mean viability set to 100 %)
$C_1 – C_8$ = TEST CHEMICAL at <u>eight</u> concentrations (C1 = highest, C8 = lowest)
b = BLANKS (contain **no** cells)

4.2.3.2 ***<u>Reporting Test Chemical Data (Phase Ib)</u>***

<u>Biweekly Reports</u>: Each testing facility will provide a biweekly progress report to the designated contacts (See Addendum I). These reports will be in electronic format (i.e., email with attachments) and will include raw and interim data as the study progresses. The Management Team will in turn provide a weekly progress report addressing the Validation Study as a whole to all of the Testing Facilities. Problems and issues shall be resolved in this manner.

<u>Draft Report</u>: At the conclusion of Phase Ib, a draft report of the Phase Ib test chemical data shall be provided by the Study Director to the designated contacts. The draft report (entitled: *In Vitro Validation Study – Phase Ib: Training Phase for Cytotoxicity Study of Three Coded Chemicals in Rodent and Human Cell Systems*) shall include everything noted in Addendum I (Draft Report – Phase Ib). If the Phase Ib data does not meet test acceptance criteria, then the Management Team (through the designated contacts) will work with the Test Facility and lead laboratory to identify problems and make corrections as needed. Once unresolved issues have been resolved, the Validation Study will proceed. The Validation Study will advance to Phase II once all participating laboratories have submitted acceptable draft reports. The draft report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the unaudited Study Workbook pages may be submitted as attachments in hard copy format.

Final Report: Once the draft report provides data that meets test acceptance criteria, then the Project Coordinator shall inform the Study Director to prepare a Quality Assurance audited final report for Phase Ib. The final report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the audited Study Workbook pages may be submitted in hard copy format as an attachment to the report. The final report will not need to be completed to continue to Phase II. (See Validation Study timelines in **Section 4.1** and Report submission timelines in **Section 4.5**.)

4.2.4 *Criteria for Advancing to Phase II*

If there is excessive variation of ICx data within or among laboratories involved in the Validation Study, the lead laboratory for each method shall assist the Management Team (through the designated contacts) to determine the cause and recommend appropriate actions needed to reduce the variation. The Statement of Work, Test Method Protocols, and SOPs shall be revised if necessary, and testing repeated until acceptable proficiency is achieved. The Management Team will decide when all laboratories will advance to the next phase of the Validation Study. A teleconference shall be held with all of the appropriate participants of the Validation Study and the Management Team will relate information concerning the advancement of the Validation Study.

4.3 Phase II

4.3.1 *Study Procedures*

Phase II of this Validation Study is the qualification phase. This phase requires testing nine blinded/coded chemicals in the same *in vitro* cytotoxicity assays and in the same concentration-response fashion as in Phase Ib. After a range-finding assay is completed, the concentration-response experiment for each chemical shall be performed three times, once each on three different days. Laboratories will **calculate IC_{20}, IC_{50}, and IC_{80} values in μg/ml,** calculate confidence limits for each value, and report this and all raw data to the Study Management Team through the designated contacts.

4.3.1.1 Reporting Test Chemical Data (Phase II)

Biweekly Reports: Each testing facility will provide a biweekly progress report to the designated contacts (See Addendum I). These reports will be in electronic format (i.e., email with attachments) and will include raw and interim data as the study progresses. The Management Team will in turn provide a weekly progress report addressing the Validation Study as a whole to all of the Testing Facilities. Problems and issues shall be resolved in this manner.

Draft Report: At the conclusion of Phase II, a draft report of the Phase II test chemical data shall be provided by the Study Director to the designated contacts. The draft report (entitled: *In Vitro Validation Study – Phase II: Qualification Phase for Cytotoxicity Study of Nine Coded Chemicals*) shall include everything noted in Addendum I (Draft Report – Phase II). If the Phase II data does not meet test acceptance criteria, then the Management Team (through the designated contacts) will work with the Test Facility and lead laboratory to identify problems and make corrections as needed. Once unresolved issues have been resolved, the Validation Study will proceed. The Validation Study will advance to Phase III once all participating laboratories have submitted acceptable draft reports. The draft report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the unaudited Study Workbook pages may be submitted as attachments in hard copy format.

<u>Final Report</u>: Once the draft report provides data that meets test acceptance criteria, then the Project Coordinator shall inform the Study Director to prepare a Quality Assurance audited final report for Phase II. The final report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the audited Study Workbook pages may be submitted in hard copy format as an attachment to the report. The final report will not need to be completed to continue to Phase III. (See Validation Study timelines in **Section 4.1** and Report submission timelines in **Section 4.5**.)

Any solubility problems/issues with the test chemicals shall be addressed by the lead laboratory and Management Team (through the designated contacts) and resolved at the end of Phase II before proceeding to Phase III.

4.3.2 *Criteria for Advancing to Phase III*

If there is excessive variation of ICx data within or among laboratories in the Validation Study, the lead laboratory/testing facility shall assist the Management Team (through the designated contacts) to determine the cause and recommend appropriate actions needed to reduce the variation. The Statement of Work, Test Method Protocols, and SOPs shall be revised if necessary and testing repeated until acceptable proficiency and reproducibility is achieved in all participating laboratories. The Management Team will decide when all laboratories will advance to the next phase of the Validation Study. A teleconference shall be held with all of the appropriate participants of the Validation Study and the Management Team will relate information concerning the advancement of the Validation Study.

4.4 Phase III

4.4.1 *Study Procedures*

Phase III of this Validation Study requires testing 60 blinded/coded chemicals in the same manner as in Phases I and II (i.e., in the *in vitro* cytotoxicity assays in a concentration-response fashion with <u>two - three replicate assays</u> [see Figure 2] after completing a range-finding assay for each chemical). ***The definitive number of replicate assays will be determined based on recommendations of the Management Team and projected costs for doing replicates (see Section 1.4).*** Laboratories will **calculate IC_{20}, IC_{50}, and IC_{80} values in µg/ml,** calculate confidence limits for each value, and report this and all raw data to the Study Management Team through the designated contacts.

4.4.1.1 ***Reporting Data (Phase III)***

Biweekly Reports: Each testing facility will provide a biweekly progress report to the designated contacts of the Management Team (See Addendum I). These reports will be in electronic format (i.e., email with attachments) and will include raw and interim data as the study progresses. The Management Team will in turn provide a weekly progress report addressing the Validation Study as a whole to all of the Testing Facilities. Problems and issues shall be resolved in this manner.

Draft Report: At the conclusion of Phase III, a draft report of the Phase III test chemical data shall be provided by the Study Director to the designated contacts. The draft report (entitled: *In Vitro Validation Study – Phase III: Cytotoxicity Study of 60 Coded Chemicals in Rodent and Human Cell Systems*) must include everything noted in Addendum I Draft Report – Phase III). If the Phase III data does not meet test acceptance criteria, then the Management Team (through the designated contacts) will work with the Test Facility and lead laboratory to identify problems and make corrections as needed. Once unresolved issues have been resolved, the Validation Study will proceed. The draft report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the unaudited Study Workbook pages may be submitted as attachments in hard copy format.

Final Report: Once the draft report provides data that meets test acceptance criteria, then the Project Coordinator shall inform the Study Director to prepare a Quality Assurance audited final report for Phase III. The final report shall be submitted in email and five (5) hard copy formats. File attachments in email shall be submitted in Microsoft® Word (or equivalent) and Excel format and all email correspondence shall be copied to the designated contacts. Copies of the audited Study Workbook pages may be submitted in hard copy format as an attachment to the report. (See Validation Study timelines in **Section 4.1** and Report submission timelines in **Section 4.5**.)

4.4.2 *Criteria for Completion of Phase III*

Phase III will be complete once all of the test chemicals (60) have been tested and the Study Director provides a final report to the designated contacts. The Validation Study will be complete (for all Testing Facilities) after the Study Management Team has received final reports from each Testing Facility and has statistically analyzed all of the data provided by all Testing Facilities.

4.5 Report Submission Timelines

4.5.1 *Draft Reports*

Draft reports for each phase shall be submitted to the Management Team through the designated contacts as per **Section 4.1.1**. The Management Team will respond to the Test Facility within two – four weeks after receipt of the report. If data are acceptable, then the Management Team (through the designated contacts) will instruct the Test Facility to continue to the next phase (teleconference with all participants). If the data do not meet the criteria and adjustments to the Validation Study are needed, a new timeline will be created and relayed to the Test Facility.

4.5.2 *Final Report*

Once the Management Team (through the designated contacts) declares to a Test Facility that the Validation Study testing phase is complete, then the Test Facility shall provide a final report (electronic and hard copy) for the identified phase of the Validation Study to the Management Team through the designated contacts as per **Section 4.1.1**.

5.0 IDENTIFICATION OF TEST CHEMICALS AND CONTROL SUBSTANCES

The NIEHS/NTP designated contractor (BioReliance) will supply all test chemicals and the positive control to all Testing Facilities. Phase I chemicals will be shipped as a unit as will the Phase II chemicals. Phase III chemicals will be shipped as one unit of 60 chemicals. The Management Team will have all pertinent information for each chemical (e.g., purity, CAS #, supplier, etc.) and will make all decisions concerning any questions about or problems/issues with the chemicals.

5.1 Test Chemicals

5.1.1 *Range of Toxicities*

The chemicals proposed for the Validation Study are representative of a range of toxicities and are relevant with regard to human exposure potential. The test chemicals will represent each of the Globally Harmonized System (GHS) classification groups for rat oral LD50s: ≤ 5 mg/kg, $>5 \leq 50$ mg/kg, $>50 \leq 300$ mg/kg, $>300 \leq 2000$ mg/kg, $>2000 \leq 5000$ mg/kg, and >5000 mg/kg (OECD, 2001).

5.1.2 *Receipt of Chemicals*

Test chemicals will be packaged so as to minimize damage during transit and will be shipped to the Testing Facility according to proper regulatory procedures. Chemicals are to be packaged and shipped so as to conceal their identities. The Study Management Team and the Testing Facility shall be notified by the contractor (BioReliance) when the test chemicals are shipped so as to prepare for receipt.

Upon receipt at the facility, the test chemicals shall be stored in appropriate storage conditions as per recommendations provided by the contractor (BioReliance). The Testing Facility shall immediately notify the Project Coordinator and the contractor about receipt of chemicals. The blinded/coded test chemicals as well as a sealed health and safety information package will be shipped to the Safety Officer. The Safety Officer shall retain the package and pass the test chemicals to the Study Director. The package will contain necessary information about the chemical hazards and provide instructions for emergency actions. A disclosure key for identifying test chemicals by code will also be included. At the end of the Validation Study, the Safety Officer shall return the unopened health and safety package to the contractor (BioReliance) who supplied the chemicals (through the designated contacts). If the health and safety package must be opened by the laboratory, the Safety Officer shall immediately notify the designated contacts.

If regulatory transportation requirements dictate that each package must display a list of the chemicals it contains on the outside of the package, the list can be removed by shippers before delivery to the participating Testing Facility. If shippers have not removed this information, the Safety Officer shall remove it prior to passing the chemicals to the Study Director.

5.1.3 *Test Chemical Information for the Study Director*

Each test chemical will be accompanied by data sheets giving a minimum of essential information, including color, odor, physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions (which will be the same for each chemical). The Study Director shall receive this information.

5.2 Control Materials

5.2.1 *Vehicle Control (VC)*

5.2.1.1 ***3T3 NRU Assay (VC)***

Dulbecco's Modification of Eagle's Medium (DMEM) buffered with sodium bicarbonate and supplemented with (final concentrations in DMEM are quoted): 5 % NBCS, 4 mM Glutamine, 100 IU Penicillin, 100 µg/ml Streptomycin. (See specifics in Test Method Protocol) [Note: Vehicle control may also be known as negative control.]

5.2.1.2 ***NHK NRU Assay (VC)***

A modified MCDB 153 formulation such as Clonetics® Keratinocyte Basal Medium (KBM®) supplemented with**:** 0.1 ng/ml Human recombinant epidermal growth factor, 5 g/ml Insulin, 0.5 g/ml Hydrocortisone, 50 g/ml Gentamicin, 50 ng/ml Amphotericin B, 0.1 mM Calcium, 2 ml 7.5 mg/ml Bovine pituitary extract. (See specifics in Test Method Protocol) [Note: Vehicle control may also be known as negative control.]

5.2.2 *Positive Control (PC)*

Sodium Lauryl Sulfate ([SLS], CAS # 151-21-3) will be the positive control for both assays. A dose-response assay of SLS dilutions will be run in one plate for each set of test chemical assays. There will be no PC in the test chemical assay plates.

5.3 Inventory of Test Chemicals

The amount of test chemical received, the amount used for specific tests, and the amount remaining shall be documented by the Testing Facility.

5.4 Disposition of Test Chemicals

After the studies are completed, the remaining test chemicals will be returned to the contractor (BioReliance) or appropriately disposed of by the Testing Facility.

5.5 Handling of Test Chemicals

Appropriate routine safety procedures shall be followed in handling the test chemicals unless the contractor (BioReliance) otherwise specifies more cautious procedures. Test Facility personnel shall be instructed to treat all blinded/coded test chemicals as ***very hazardous and potentially carcinogenic*** and to dispose of laboratory wastes as toxic wastes. The health and safety information package provided to the Testing Facility Safety Officer shall be examined by the Testing Facility only in an emergency/need-to-know situation.

5.6 Determination of Purity, Composition, and Stability of Test Chemicals

The contractor (BioReliance) will be responsible for collecting information on the analytical purity, composition, and stability of the test chemicals and the positive control material from manufacturer and supplier documentation. The contractor will provide information on chemical homogeneity in the vehicle via solubility studies. Chemicals shall be stored in an appropriate manner as stated by the contractor.

6.0 TEST SYSTEM

All testing procedures and data analyses shall follow the Test Method Protocols and Statement of Work provided by the Management Team which are based on the NIEHS Publication # 01-4500, *Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity* (ICCVAM, 2001a).

6.1 Neutral Red Uptake (NRU) Cytotoxicity Assay

6.1.1 *Background*

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

6.1.2 *Sterility of the Test System*

All cell culture applications shall be conducted under aseptic conditions. The test system shall be deemed free of mycoplasmal, fungal, and/or bacterial contamination. The cell suppliers ship cryopreserved cells that have been tested for mycoplasma and are deemed mycoplasma-free. If mycoplasma contamination is suspected, then the Testing Facility shall have the cells tested in an appropriate manner. If mycoplasma is present, all old cells of the specific lot of cells shall be eliminated and new cell stocks shall be prepared or purchased. The presence of bacterial or fungal contamination in the cultures shall be determined by gross visual inspection during and at the conclusion of each assay. If bacterial or fungal contamination is present in the cultures, the Study Director shall determine the course of action.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY – 3T3 NRU ASSAY

7.1 Major Steps in the Performance of the Assay

BALB/c 3T3 cells are seeded into 96-well plates and maintained in culture for 24 hours (h) (~ 1 doubling period) to form a semi-confluent monolayer

⇓

Remove culture medium

⇓

Cells are then exposed to the test chemical in treatment medium over a range of 8 concentrations for 48 h exposure

⇓

Microscopic evaluation of morphological alterations

⇓

Remove treatment medium; wash once with PBS; add Neutral Red (NR) medium; incubate for 3 h.

⇓

Discard NR medium; wash once with PBS; add NR desorbing fixative

⇓

Shake plate for 20 minutes

⇓

Detect NR Absorption at optical density (OD) 540nm

⇓

Perform Neutral Red Uptake data calculations (% viability; calculations of IC_{20}, IC_{50}, and IC_{80} values)

7.2 Procedures for Conducting the Test

All testing procedures and data analyses shall follow the Test Method Protocols and Statement of Work provided by the Management Team and SOPs produced by the Testing Facility. All deviations from Statement of Work or SOPs shall be documented in the Study Workbook. The following abbreviated descriptions of the SOPs provide an overview of the assay, but must not be used in place of the formal SOPs.

7.2.1 *Cell Maintenance and Culture Procedures*

Ampules of cryopreserved BALB/c 3T3 cells are quickly thawed in a 37°C water bath. The cells are resuspended in cell culture medium and transferred to cell culture flasks. The thawed cells are incubated at 37°C in a 90 % humidified 5.0 % CO_2 atmosphere. Cells are passaged two to three times before using them in a cytotoxicity test. A fresh batch of cryopreserved cells should be thawed out approximately every two months (See **Section 7.2.1.1**). This period resembles a sequence of about 18 passages.

The cells are routinely grown as a monolayer in tissue culture grade flasks, at 37°C in a 90 % humidified atmosphere of 5.0 % CO_2 and are examined on a daily basis under a phase contrast microscope.

When cells approach a predetermined confluency, they must be detached from the flask by trypsinization, resuspended in culture medium, and counted using a hemocytometer or cell counter. After determination of cell number, the cell culture must be sub-cultured into other flasks or seeded into 96-well microtiter plates. Stocks of BALB/c 3T3 cells are prepared in a medium with DMSO as a cryoprotective agent and stored in sterile, freezing tubes in a liquid nitrogen freezer for long-term storage.

7.2.1.1 ***Cryopreserved Lots of Cells***

After the initial establishment of the 3T3 cells in culture from an ampule of cryopreserved cells (from the cell supplier), laboratory personnel shall grow enough cells for cryopreservation in a number of freeze tubes (e.g., 10 – 20 tubes). These tubes will form the stock pool from which subsequent cultures will be established for use in the assays (See **Section 7.2.1**).

7.2.1.2 ***Determination of Cell Doubling Time***

A cell doubling time procedure shall be performed on the initial lot of cells that will be used in the first cell culture assays of Phase Ia of the Validation Study. The doubling time only needs to be determined again if there is a change in the lot of cells used. The Test Method Protocol will provide the basic procedures for this determination.

8.0 EXPERIMENTAL DESIGN AND METHODOLOGY – NHK NRU ASSAY

8.1 Major Steps in the Performance of the Assay

NHK cells are seeded into 96-well plates and maintained in culture for 24 – 72 hours (h) to form a semi-confluent (30 – 50 %) monolayer

⇓

Remove culture medium

⇓

Cells are then exposed to the test chemical in treatment medium over a range of 8 concentrations for 48 h exposure

⇓

Microscopic evaluation of morphological alterations

⇓

Remove treatment medium; wash once with PBS; add Neutral Red (NR) medium; incubate for 3 h.

⇓

Discard NR medium; wash once with PBS; add NR desorbing fixative

⇓

Shake plate for 20 minutes

⇓

Detect NR Absorption at optical density (OD) 540nm

⇓

Perform Neutral Red Uptake data calculations (% viability; calculations of IC_{20}, IC_{50}, and IC_{80} values)

8.2 Procedures for Conducting the Test

All testing procedures and data analyses shall follow the Test Method Protocols and Statement of Work provided by the Management Team and SOPs produced by the Testing Facility. All deviations from the Statement of Work or SOPs shall be documented in the Study Workbook. The following abbreviated descriptions of the SOPs provide an overview of the assay, but must not be used in place of the formal SOPs. Information specific to the keratinocytes as provided by the supplier (e.g., Clonetics) shall be considered when preparing SOPs.

8.2.1 *Cell Maintenance and Culture Procedures*

Ampules of cryopreserved NHK cells are quickly thawed in a 37°C water bath. The cells are resuspended in cell culture medium and transferred to cell culture flasks. The thawed cells are incubated at 37°C in a 90 % humidified 5.0 % CO_2 atmosphere. NHK cells will be sustained in culture through only one passage after establishing cells in culture.

The cells are routinely grown as a monolayer in tissue culture grade flasks, at 37°C in a 90 % humidified atmosphere of 5.0 % CO_2 and are examined on a daily basis under a phase contrast microscope.

When cells approach a predetermined confluency, they must be detached from the flask by trypsinization, resuspended in culture medium, and counted using a hemocytometer or cell counter. After determination of cell number, the cell culture must be seeded into 96-well microtiter plates.

8.2.1.1 ***Determination of Cell Doubling Time***

A cell doubling time procedure shall be performed on the initial lot of cells that will be used in the first cell culture assays of Phase Ia of the Validation Study. The doubling time only needs to be determined again at the initiation of the cells in culture if there is a change in the lot of cells used. The Test Method Protocol will provide the basic procedures for this determination.

9.0 PREPARATION AND DELIVERY OF TEST CHEMICAL

9.1 Preparation of Test Chemical

The test chemical must be freshly prepared immediately prior to use. All chemicals shall be weighed on a calibrated balance (including liquid test chemicals) and added to the appropriate solvent (**Section 9.1.1**). Test chemicals must be at room temperature before dissolving and diluting. Preparation under red or yellow light may be necessary, if rapid photodegradation is likely to occur. The solutions must not be cloudy nor have noticeable precipitate.

The following hierarchy (culture medium, DMSO, ethanol) shall be followed for dissolving the test chemical.

9.1.1. *Dissolving the Test Chemical*

9.1.1.1 ***Treatment Medium/Routine Culture Medium)***

a) Dissolve test chemical in Treatment Medium [3T3] or Routine Culture Medium [NHK] (See Test Method Protocols).
b) Gently mix. Vortex (1 –2 minutes).
c) If test chemical hasn't dissolved, use sonication (up to five minutes).
d) If sonication doesn't work, then warm solution to 37°C.

9.1.1.2 ***DMSO***

If the test chemical doesn't dissolve in the Treatment Medium/Routine Culture Medium, then follow steps a) through d) in **Section 9.1.1.1** using DMSO instead of Treatment Medium/Routine Culture Medium.

9.1.1.3 ***Ethanol***

If the test chemical doesn't dissolve in DMSO, then follow steps a) through d) in **Section 9.1.1.1** using ethanol instead of DMSO.

9.1.2. *Test Chemical Solubility*

Each test chemical will be prepared such that the highest test concentration in each range finding experiment is 100 mg/ml (100,000 μg/ml) in culture medium (10 mg/ml [10,000 μg/ml] in culture medium if DMSO or ethanol is used as a solvent). If the range finding experiment shows that 100,000 μg/ml is not high enough for the IC_{50} values in the range to meet the acceptance criteria, then higher concentrations will be used for the definitive experiment.

Solubility of the test chemical will be determined by following a modified version of EPA Product Properties Test Guidelines OPPTS 830.7840 (EPA, 1998). (See Test Method Protocols).

Dissolve the test chemical (at 200-fold the desired final concentration in the case of solvents) in an appropriate solvent. The final solvent (i.e., DMSO or ethanol) concentration should be kept at a constant level of no more than 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations (i.e., each concentration shall have the same amount of solvent). This means the test chemical is dissolved in the vehicle first, and then 1 part of this stock solution is added to 199 parts of sterile pre-warmed (37°C) medium. Check carefully to determine whether the chemical is still dissolved after the transfer from solvent stock solution to medium, and reduce the highest test concentration, if necessary.

The test chemicals selected for the Validation Study will be soluble. If an appropriate concentration cannot be achieved for the range finding experiments, then the Study Director shall contact the Study Management Team through the designated contacts. Prior to initiating any test chemical assay (and after performing solubility tests on the chemicals), the Study Director shall contact the Study Management Team (through the designated contacts) for discussion of the solvent to be used for test chemical application. The Management Team will provide direct guidance to the Study Director as to which solvent will be used for the assay.

9.1.3 *pH of Dilutions*

Measure the pH (using pH paper) of the highest concentration of the test chemical to be tested in the assay. Document the pH and note the color of the medium. Do not adjust the pH of the test chemical solutions.

9.2 Delivery of Test Chemical

The test chemical will be administered by direct addition (pipetting) to the 96-well microtiter plate with a vehicle compatible with the test system. The cells will be exposed to the test chemical for approximately 48 hours..

[Note: The 3T3 and NHK cells in the 96-well plate will have freshculture medium on the cells immediately prior to dosing with the test chemical. Each well will receive a volume of test chemical concentration therefore diluting the concentration by a factor of two.]

9.3 Range Finder Experiment

Test eight concentrations of the test chemical by diluting the stock solution with a constant factor. The initial dilution series will be log dilutions (i.e., 1:10, 1:100, 1:1000, etc.). If this dilution series meets test acceptance criteria (**Section 11.0**), then the range finding experiment dilutions can be used as the actual dilutions in the separate definitive test chemical experiment. If the dilution factor needs to be adjusted for the actual definitive experiment, then follow dilution schemes provided in **Section 9.4.**

9.4 Test Chemical Dilutions

a) A factor of $\sqrt[2]{10} = 3.16$ could be used for covering a large range:
(e.g., 1 ⇒3.16 ⇒10 ⇒31.6 ⇒100 ⇒316 ⇒1000 ⇒3160 µg/ml).

b) The simplest geometric concentration series (i.e., constant dilution / progression factor) are **dual geometric series** (e.g., a factor of 2). These series have the disadvantage of numerical values that permanently change between logs of the series:
(e.g., *log0*-2, 4, 8; *log1*- 16, 32, 64; *log2*- 128, 256, 512; *log3*- 1024, 2048,).

c) The **decimal geometric series**, first described by Hackenberg and Bartling (1959) for use in toxicological and pharmacological studies, has the advantage that independent experiments with wide or narrow dose factors can be easily compared because they share identical concentrations. Furthermore, under certain circumstances, experiments can even be merged together:

EXAMPLE:

10						**31.6**						**100**
10				**21.5**				**46.4**				**100**
10		**14.7**		**21.5**		**31.6**		**46.4**		**68.1**		**100**
10	**12.1**	**14.7**	**17.8**	**21.5**	**26.1**	**31.6**	**38.3**	**46.4**	**56.2**	**68.1**	**82.5**	**100**

The dosing factor of **3.16** (= $\sqrt[2]{10}$) divides a log into two equidistant steps, a factor of **2.15** (= $\sqrt[3]{10}$) divides a decade into three steps. The factor of **1.47** (= $\sqrt[6]{10}$) divides a log into six equidistant steps, and the factor of **1.21** (= $\sqrt[12]{10}$) divides the log into 12 steps.

For an easier biometrical evaluation of several related concentration response experiments use decimal geometric concentration series rather than dual geometric series. The technical production of decimal geometric concentration series is simple. An example is given for factor 1.47:

Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

d) Determine which test chemical concentration is closest to the IC_{50} value (e.g., 50 % cytotoxicity). Use that value as the central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

10.0 DATA COLLECTION

10.1 Nature of Data to be Collected

After the test is performed and the NR is desorbed from the cells, measure the absorption of the resulting colored solution at 540 nm in a microtiter spectrophotometric plate reader, using the blanks as a reference. Save raw data in the file format provided by the Study Management

Team (Microsoft® Excel template [Addendum II]) for further analysis of the concentration-response (% viability calculations). Data from the OD analyses will be used for the calculation of IC_{20}, IC_{50}, and IC_{80} values (μg/ml).

10.2 Type of Media Used for Data Storage

Originals of the raw data (the Study Workbook and computer printouts of absorbance readings from the plate reader) and copies of other raw data such as instrument logs shall be collected and archived at the end of the Validation Study (under the direction of the Study Director), according to GLP-compliant procedures. The electronic files of plate reader data and any derived data shall be saved, and a backup of these electronic files shall be produced and maintained. Calculations to convert the raw data to derived data shall be performed using Microsoft® Excel (Addendum II). The derived assay data that are stored electronically shall be periodically copied, and backup files shall be produced and maintained.

10.3 Documentation

Original raw data that shall be collected shall include but are not limited to the following:

- Data recorded in the Study Workbook, which shall consist of all recordings of all activities related to preparing the 3T3 and NHK cultures and test chemicals and performing the NRU assay;
- Computer printouts of absorbance readings from the plate reader spectrophotometer;
- Other data collected as part of GLP compliance
  - Equipment logs
  - Equipment calibration records
  - Test chemical logs
  - Cryogenic freezer inventory logs
  - Cell culture media preparation logs

Addendum IV provides examples of equipment logs.

11.0 ACCEPTANCE CRITERIA FOR NRU ASSAYS

11.1 Test Acceptance Criteria

The test method protocols provide the definitive test acceptance criteria which include a specific mean OD_{540} of all vehicle controls, a set percent difference of the mean OD_{540} between two sets of vehicle controls, and a set range of the IC_{50} for SLS.

The Study Director shall decide if a test meets acceptance criteria and the Study Management Team will make decisions concerning re-testing of test chemicals.

11.2 IC_{50} Acceptance Criteria

The IC_{50} derived from the concentration-response assays shall be based on at least <u>three responses</u> that are ≥ 10 % and ≤ 90 % inhibition of NRU. If this is not the case, and the concentration progression factor can be easily reduced, the experiment shall be rejected and a retest shall be performed with a smaller progression factor.

The raw data output from the plate reader shall be converted into the derived data using Microsoft® Excel (Addendum II). The PC and VC from each assay shall be compared to the acceptable historical ranges as noted. If the assay is found to be valid by these criteria, then the data from that assay is considered to be acceptable. If the PC or VC values are not acceptable, the assay shall be repeated. Results of all assays, acceptable and failed, shall be forwarded to the designated contacts via the previously identified reports.

12.0 EVALUATION OF TEST RESULTS

12.1 Cell Viability Determination

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six replicate values (minimum of four acceptable replicate wells) per test concentration. The Study Director shall determine if any wells do not meet expected performance criteria through visual microscopic evaluation (i.e., experimental conditions within the wells are compromised due to situations such as insufficient cell population, mechanical disruption of the monolayer, etc.). The Study Director shall decide if any of the wells of the plate need to be excluded from data analyses. If a concentration does not have a minimum of four replicate wells, then data from that concentration will not used. The test may still be acceptable if all criteria in **Section 11.1** are met (e.g., the IC_{50} derived from the concentration-response assays is backed by at least <u>three responses</u> ≥ 10 % and ≤ 90 % inhibition of NRU.) If any wells have bacterial or fungal contamination, the entire plate must be repeated.

The cell viability value is compared with the mean NRU of all VC values (provided VC values have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each chemical tested will span the range of no effect up to total inhibition of cell viability.

12.2 IC_X Determination

The concentration of a test chemical reflecting a 20 %, 50 %, and 80 % inhibition of cell viability (i.e., the IC_{20}, IC_{50}, and IC_{80}) is determined from the concentration-response and shall be done by applying a Hill function to the concentration-response data. It will not be necessary for the Testing Facilities to derive the equation. The Testing Facility shall calculate the IC_{20}, IC_{50}, and IC_{80} values for each test chemical and the confidence limits for each value using statistical software (e.g., GraphPad PRISM® 3.0) specified by the Study Management Team. In addition, the Study Management Team shall provide guidelines for calculating ICx values and confidence limits. The Testing Facility shall report data using at least three (3) significant figures and shall forward the results from each assay to the Study Management Team/biostatistician through the designated contacts in electronic format and hard copy upon completion of all testing. The Study Management Team will be directly responsible for the statistical analyses of the Validation Study data.

Hill function: a four-parameter logistic mathematical model relating the concentration of test chemical to the response being measured in a sigmoidal shape.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{logIC50} - \text{X})\text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logIC50 is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

13.0 DRAFT AND FINAL REPORTS

A draft report shall be submitted to the Management Team through the designated contacts at the completion of each study phase (Ia, Ib, II, III). A Final Report for each phase of the Validation Study shall be prepared by the Testing Facility, signed by the Study Director, and provided to the Management Team through the designated contacts upon acceptance of data provided in the corresponding draft report. The submitted results shall accurately describe all methods used for generation and analysis of the data, provide a complete record of the preparation of test chemicals, and present any relevant data necessary for the assessment of the results (See Addendum I).

14.0 RECORDS AND ARCHIVES

At the end of the Validation Study, the original raw and derived assay data, as well as copies of other raw data not exclusive to this Validation Study (instrument logs, calibration records, facility logs, etc.), shall be submitted to NIEHS/NICEATM for storing and archiving according to the facility's SOP and in compliance with GLP Standards.

Originals of all raw and derived data, or copies where applicable, shall be stored and archived at NIEHS/NICEATM.

Copies of all raw and derived data shall be stored and archived at the participating Testing Facility for at least five years after completion of the Validation Study.

15.0 ALTERATIONS OF THE STATEMENT OF WORK

No changes in the Statement of Work shall be made without the consent of the Management Team. A Statement of Work Amendment detailing any change(s) and the basis for the change(s) shall be approved and prepared by the Study Director, and the amendment shall be signed and dated by the Study Director and the NIEHS representative. The amendment shall be retained with the original Statement of Work.

16.0 REFERENCES

Clonetics Normal Human Keratinocyte Systems Instructions for Use, AA-1000-4-Rev.03/00. (http://www.clonetics.com).

Cooper-Hannan, R., J.W. Harbell, S. Coecke, M. Balls, G. Bowe, M., Cervinka, R. Clothier, F. Hermann, L.K. Klahm, J. de Lange, M. Liebsch and P. Vanparys. 1999. The Principles of Good Laboratory Practice: application to in vitro toxicology studies. The Report and Recommendations of the ECVAM Workshop 37. ATLA. 27 (4). July/August. Pp. 539 – 577.

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NICEATM (The National Toxicology Program [NTP] Interagency Center for the Evaluation of Alternative Toxicological Methods). 2001. Test Method Protocol for the Normal Human Keratinocyte [NHK] Neutral Red Uptake Cytotoxicity Test. A Test for Basal Cytotoxicity for an *In Vitro* Validation Study.

OECD (Organisation for Economic Co-operation and Development). 2001. Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures as Endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals in November 1998, Part 2, p.21. OECD, Paris. http://www.oecd.org/ehs/class/HCL6htm.

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OECD (Organisation for Economic Co-operation and Development). 1992. Environment Monograph No 50, Series on Principles of Good Laboratory Practice and Compliance Monitoring: Number 6, The Application of the GLP Principles to Field Studies. Paris, France: OECD.

Spielmann, H., S. Gerner, S. Kalweit, R. Moog, T. Wirnserberger, K. Krauser, R. Kreiling, H. Kreuzer, N.P. Luepke, H.G. Miltenburger, N. Müller, P. Murmann, W. Pape, B. Siegmund, J. Spengler, W. Steiling, and F.J. Wiebel. 1991. Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. Toxicol. *In Vitro* 5: 539-542.

Triglia, D., P.T. Wegener, J. Harbell, K. Wallace, D. Matheson, and C. Shopsis. 1989. Interlaboratory validation study of the keratinocyte neutral red bioassay from Clonetics Corporation. In *Alternative Methods in Toxicology*, Volume 7. A.M. Goldberg, ed., pp. 357-365. Mary Ann Liebert, Inc., New York.

17.0 APPROVAL OF STATEMENT OF WORK

Sponsor Representative	**Date**
Testing Facility Management	**Date**

ADDENDUM I

SUGGESTED REPORT FORMAT

<u>TITLE PAGE</u>

- **Study Title**
  - Draft/Final Report 1: *In Vitro Validation Study – Phase Ia: Development of a Positive Control Database in Rodent and Human Cell Systems*
  - Draft/Final Report 2: *In Vitro Validation Study – Phase Ib: Training Phase for Cytotoxicity Study of Three Coded Chemicals in Rodent and Human Cell Systems*
  - Draft/Final Report 3: *In Vitro Validation Study – Phase II: Qualification Phase for Cytotoxicity Study of Nine Coded Chemicals in Rodent and Human Cell Systems*
  - Draft/Final Report 4: *In Vitro Validation Study – Phase III: Cytotoxicity Study of 60 Coded Chemicals in Rodent and Human Cell Systems*

- ***In Vitro* Assay**
  Identify the assays: 3T3 NRU and NHK NRU

c) **Test Articles**
  - Draft/Final Report 1: (Phase Ia) identify the positive control chemical
  - Draft/Final Report 2: (Phase Ib) identify the three (3) test chemicals
  - Draft/Final Report 3: (Phase II) identify the nine (9) test chemicals
  - Draft/Final Report 4: (Phase III) identify the sixty (60) test chemicals

- **Authors**
- **Study Completion Date**
- **Testing Facility**
- **Validation Study Number/Identification**

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

SIGNATURE PAGE

- **Validation Study Initiation Date**
  Date Protocol was signed by Study Director
- **Initiation Date of Laboratory Studies**
  Actual laboratory start date
- **Validation Study Completion Date**
  Date report signed by Study Director
- **Sponsor Representative**
  The National Institute of Environmental Health Sciences (NIEHS)
  The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

  NICEATM
  79 T.W. Alexander Drive
  Bldg. 4401, MD-EC-17
  3rd Floor, Room 3126
  P.O. Box 12233
  Research Triangle Park, NC 27709
- **Study Management Team Representatives**
  Judy Strickland, Ph.D. (Project Coordinator)
  Michael Paris (Assistant Project Coordinator)
- **Testing Facility**
  Name and address
- **Archive Location**
  Name and address
- **Study Director**
  Name and signature and date
- **Key Personnel**
  Laboratory technicians, QA Director, Safety Officer
- **Facility Management**
  Name
- **Scientific Advisor**
  Name

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

TEST CHEMICAL RECEIPT PAGE

Test Chemical Receipt Reporting Template for *In Vitro* Validation Study

Test Facility Test Chemical Identification Number	Sponsor Test Chemical Identification Number	Test Chemical Physical Description	Storage Conditions	Test Chemical Receipt Date	Test Chemical Received By	Comments

ADDENDUM I (cont.)
SUGGESTED REPORT FORMAT
DRAFT/FINAL REPORT 1
In Vitro Validation Study – Phase Ia: Development of a Positive Control Database in Rodent and Human Cell Systems

- **Table of Contents**

- **Objectives:** The reports shall provide specific objectives

- **Description of the Test System Used:** Description of 3T3 NRU assay and the NHK NRU assay

- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for range finding experiments

- **Narrative Description of the Assays:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical will be included in the description. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report as attachments. The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.

- **Statement Signed by the Study Director:** Confirm that the Validation Study was conducted in compliance with GLP (or indicating where the Study deviated from GLP), or for non GLP-compliant laboratories, confirm that the Validation Study adhered to the spirit of GLP. Confirm that the report fully and accurately reflects the raw data generated in the Validation Study.

- **Quality Assurance Statement: (For Final Report only)**
- For GLP-Compliant Laboratories: QA Statement identifying: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study.
- For Non GLP-Compliant Laboratories: A statement from the Testing Facility shall be included with the Final Report of Phase III. This statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study and provide assurance that all testing was done in the spirit of GLP.

- **Data Analysis:** (for each NRU assay) calculate the % viability for each positive control chemical concentration (eight concentrations per assay); determine the IC_{50} values for the positive control in each assay; follow guidelines/procedures in Statement of Work and Test Method Protocols.

- **Other Information: (All copies of printouts, documents, and spreadsheets will be noted as exact duplicates of the data.)**
  - Copies of spectrometric plate reader raw data
  - Copies of the completed Microsoft® Excel spreadsheets (Addendum II) used for calculation of cytotoxicity values
  - Copies of data pages showing IC_{50} calculations for the positive control
  - Copy of the protocols
  - Deviations to the protocols, SOPs, and Statement of Work
  - Revisions/amendments to the protocols, SOPs, and Statement of Work

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

DRAFT/FINAL REPORT 2

In Vitro Validation Study – Phase Ib: Training Phase for Cytotoxicity Study of Three Coded Chemicals in Rodent and Human Cell Systems

- **Table of Contents**

- **Objectives:** The reports shall provide specific objectives

- **Description of the Test System Used:** Description of 3T3 NRU assay and the NHK NRU assay

- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for range finding experiments

- **Narrative Description of the Assays:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical shall be included in the description. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report as attachments. The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.

- **Statement Signed by the Study Director**: Confirm that the Validation Study was conducted in compliance with GLP (or indicating where the Study deviated from GLP), or for non GLP-compliant laboratories, confirm that the Validation Study adhered to the spirit of GLP. Confirm that the report fully and accurately reflects the raw data generated in the Validation Study.

- **Quality Assurance Statement: (For Final Report only)**
- For GLP-Compliant Laboratories: QA Statement identifying: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study.
- For Non GLP-Compliant Laboratories: A statement from the Testing Facility shall be included with the Final Report of Phase III. This statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study and provide assurance that all testing was done in the spirit of GLP.

- **Data Analysis:** (for each assay) calculate the % viability for the positive control and each test chemical concentration (eight concentrations per assay); determine the IC_{50} value for the positive control; determine the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each of the three (3) test chemicals.

- **Other Information: (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)**
    - Copies of spectrometric plate reader raw data

- Copies of the completed Microsoft® Excel spreadsheets (Addendum II) used for calculation of cytotoxicity values
- Copies of data pages showing IC_{50} calculations for the positive control and the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each test chemical
- Copy of the protocols
- Deviations to the protocols, SOPs, and Statement of Work
- Revisions/amendments to the protocols, SOPs, and Statement of Work

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

DRAFT/FINAL REPORT 3
In Vitro Validation Study – Phase II: Qualification Phase for Cytotoxicity Study of Nine Coded Chemicals in Rodent and Human Cell Systems

- **<u>Table of Contents</u>**

- **<u>Objectives</u>:** The reports shall provide specific objectives

- **<u>Description of the Test System Used</u>:** Description of 3T3 NRU assay and the NHK NRU assay

- **<u>Summary of the Findings</u>:** Referenced to the raw data where appropriate; Include all information for range finding experiments

- **<u>Narrative Description of the Assays</u>:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical will be included in the description. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report as attachments. The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.

- **<u>Statement Signed by the Study Director</u>:** Confirm that the Validation Study was conducted in compliance with GLP (or indicating where the Study deviated from GLP), or for non GLP-compliant laboratories, confirm that the Validation Study adhered to the spirit of GLP. Confirm that the report fully and accurately reflects the raw data generated in the Validation Study.

- **<u>Quality Assurance Statement: (For Final Report only)</u>**
- <u>For GLP-Compliant Laboratories</u>: QA Statement identifying: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study.
- <u>For Non GLP-Compliant Laboratories</u>: A statement from the Testing Facility shall be included with the Final Report of Phase III. This statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study and provide assurance that all testing was done in the spirit of GLP.

- **<u>Data Analysis</u>:** (for each assay) calculate the % viability for the positive control and each test chemical concentration (eight concentrations per assay); determine the IC_{50} value for the positive control; determine the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each of the nine (9) test chemicals.

- **<u>Other Information</u>: (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)**
  - Copies of spectrometric plate reader raw data

- Copies of the completed Microsoft® Excel spreadsheets (Addendum II) used for calculation of cytotoxicity values
- Copies of data pages showing IC_{50} calculations for the positive control and the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each test chemical
- Copy of the protocols
- Deviations to the protocols, SOPs, and Statement of Work
- Revisions/amendments to the protocols, SOPs, and Statement of Work

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

DRAFT/FINAL REPORT 4

- ***In Vitro Validation Study – Phase III: Cytotoxicity Study of 60Coded Chemicals in Rodent and Human Cell Systems***
- **Table of Contents**

- **Objectives:** The draft report shall provide specific objectives

- **Description of the Test System Used:** Description of 3T3 NRU assay and the NHK NRU assay

- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for range finding experiments

- **Narrative Description of the Assays:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical shall be included in the description. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report as attachments. The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.

- **Statement Signed by the Study Director:** Confirm that the Validation Study was conducted in compliance with GLP (or indicating where the Study deviated from GLP), or for non GLP-compliant laboratories, confirm that the Validation Study adhered to the spirit of GLP. Confirm that the report fully and accurately reflects the raw data generated in the Validation Study.

- **Quality Assurance Statement: (For Final Report only)**
- For GLP-Compliant Laboratories: QA Statement identifying: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study.
- For Non GLP-Compliant Laboratories: A statement from the Testing Facility shall be included with the Final Report of Phase III. This statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Validation Study and provide assurance that all testing was done in the spirit of GLP.

- **Data Analysis:** (for each assay) calculate the % viability for the positive control and each test chemical concentration (eight concentrations per assay); determine the IC_{50} value for the positive control; determine the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each of the 60 (or 30) test chemicals.

- **Other Information: (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)**
  - Copies of spectrometric plate reader raw data
  - Copies of the completed Microsoft® Excel spreadsheets (Addendum II) used for calculation of cytotoxicity values

- Copies of data pages showing IC_{50} calculations for the positive control and the IC_{20}, IC_{50}, and IC_{80} values (and confidence limits) for each test chemical
- Deviations to the protocols, SOPs, and Statement of Work
- Copy of the protocols
- A list of all SOPs used by the laboratory for the assays (SOP title and laboratory identification code)
- The Statement of Work and The Test Method Protocols

ADDENDUM I (cont.)

SUGGESTED REPORT FORMAT

BIWEEKLY REPORTS

Testing Facility:

Chemicals Received:

Chemicals Tested:
3T3 NRU Assay:
NHK NRU Assay:

Solubility Determinations: (solvents used and concentrations obtained)

Range Finding Experiments: (number performed; outcomes)

Successful Tests: (number of tests and calculated IC_{20}, IC_{50}, and IC_{80} values; include Excel® spreadsheets)

Failed Tests: (number of failed tests and reasons for failure)

Problems Encountered/Resolutions:

Projected Testing Schedule:

ADDENDUM II
EXCEL SPREADSHEET TEMPLATE FOR ASSAY DATA

Test Facility	dfdgs			Cell Line/Type	3T3
Chemical Code	4567			Vehicle Contol	0.5% DMSO
Plate ID	qa789				
Date Read	######				

Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
C	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
D	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
E	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
F	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
G	Blank	VC 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	VC2	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Plate Data

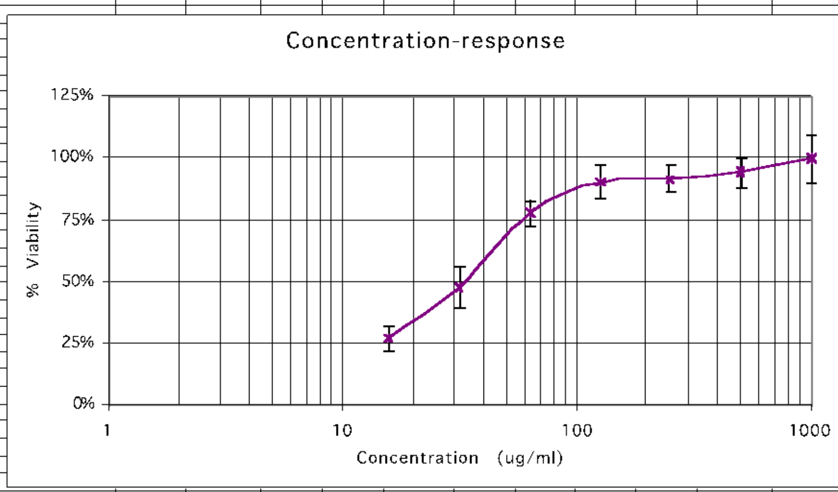
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.004	0.006	0.036	0.004	0.028	0.019	0.023	0.029	0.012	0.003	0.004	0.011
B	0.009	0.832	0.832	0.855	0.780	0.755	0.693	0.419	0.265	0.052	0.832	0.008
C	0.014	0.894	0.894	0.916	0.884	0.83	0.73	0.368	0.213	0.105	0.935	0.012
D	-0.006	0.918	0.918	0.87	0.914	0.835	0.806	0.450	0.270	0.098	0.918	0.009
E	-0.004	0.915	0.915	0.826	0.903	0.879	0.73	0.591	0.295	0.086	0.915	0.015
F	-0.004	1.098	1.098	0.984	0.814	0.952	0.746	0.436	0.201	0.151	1.098	0.014
G	0.016	0.948	0.948	0.845	0.842	0.832	0.663	0.431	0.319	0.09	0.89	0.015
H	-0.001	-0.006	0.017	-0.005	0.009	0.004	0.002	0.014	-0.013	-0.003	-0.061	0.012

Mean blank OD: 0.0068

Corrected OD = OD- mean blank OD

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.825	0.825	0.848	0.773	0.748	0.686	0.412	0.258	0.045	0.825	
C		0.887	0.887	0.909	0.877	0.823	0.723	0.361	0.206	0.098	0.928	
D		0.911	0.911	0.863	0.907	0.828	0.799	0.443	0.263	0.091	0.911	
E		0.908	0.908	0.819	0.896	0.872	0.723	0.584	0.288	0.079	0.908	
F		1.091	1.091	0.977	0.807	0.945	0.739	0.429	0.194	0.144	1.091	
G		0.941	0.941	0.838	0.835	0.825	0.656	0.420	0.312	0.083	0.883	
H												

	blanks	Vehicle Control 1	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Conc 6	Conc 7	Conc 8	Vehicle Control 2	blanks
Concentration [µg/ml]		0	1000	500	250	125	62.5	31.25	15.625	7.2	0	
Mean Corrected OD		0.927	0.927	0.876	0.849	0.840	0.721	0.442	0.254	0.090	0.925	
SD of Mean OD	0.0158	0.089	0.089	0.058	0.053	0.065	0.049	0.075	0.046	0.032	0.089	
Corrected Mean ------	All VCs	0.926										
% Viability = Mean Corrected OD/Mean Corrected VC	100%	100%	100%	95%	92%	91%	78%	48%	27%	10%	100%	
SD (% Viability) = SD OD/Mean OD All VCs		10%	10%	6%	6%	7%	5%	8%	5%	3%	10%	
%CV = SD/mean OD*100	9%	9.6%	9.6%	6.6%	6.3%	7.7%	6.8%	17.0%	18.1%	35.7%	9.7%	
Mean Vehicle Control - VC1 (%)		-0.15%										
Mean Vehicle Control - VC2 (%)		0.15%										



ADDENDUM III

SUGGESTED STANDARD TEST REPORTING TEMPLATE FOR *IN VITRO* VALIDATION STUDY WORKBOOK

TEST CHEMICAL	
Test Facility	96-Well Plate ID _______________
Chemical Code	Experiment ID ________________

PREPARATION OF TEST CHEMICAL	
Solvent _____Culture Medium _____DMSO _____Ethanol	
Highest Percent Solvent (v/v) in Dilutions _______%	Highest Concentration Tested_______μg/ml
Aids Used to Dissolve _____Vortex _____Ultra-sonicaton _____Heat to 37°C	
pH (Highest Test Concentration)___________ Media color of test chemical solutions:	
Positive Control [SLS]_________μg/ml	Vehicle Control __________% solvent

Concentration Series (μg/ml)	C1	C2	C3	C4	C5	C6	C7	C8

CELL LINE/TYPE		
Name	Supplier	From Cell Lot No.________
Total Passage No.	No. of Passages after Thawing	From:____ proliferating ____frozen

CELL CULTURE CONDITIONS		
Name of Medium	Supplier/ID	Lot No./Lab I.D.
Name of Serum	Supplier/ID	Lot No.
Serum Concentration	During Growth: _________%	During Exposure: __________%

TEST ACCEPTANCE CRITERIA			
VC: Mean Absolute OD_{540}	Mean OD =______	____Accept	____Reject
VC: Difference Between Col.2 and Col. 10	Difference =_____%	____Accept	____Reject
PC: IC_{50} of Concurrent SLS Test	IC_{50} =________μg/ml	____Accept	____Reject

TIMELINE		
Assay Start Date (cells to plates)	Application of Test Chemical Date	NRU/OD_{540} Measurement Date

ADDENDUM IV

EXAMPLES OF LABORATORY EQUIPMENT LOGS

INCUBATOR

INCUBATOR I.D.________________________

MONTH:___________ YEAR:___________ LOCATION:________________

DATE	TIME	INITIALS	CO_2 %	RH %	TEMP. (^{O}C.)	CO_2 TANK (PSI)	CO_2 TANK (NEW)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
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31							

FYRITE CHECK OF CO2:

ADDITION OF WATER:

TOTAL INCUBATOR DISINFECTION:

ADDENDUM IV (cont.)

EXAMPLES OF LABORATORY EQUIPMENT LOGS

pH METER

pH METER I.D.________________________

MONTH:___________ YEAR:___________ LOCATION:________________

DATE	TIME	INITIALS	pH STD. 7.00	pH STD. 10.00	pH STD. 4.00	pH STD. 7.40	SLOPE
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
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19							
20							
21							
22							
23							
24							
25							
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28							
29							
30							
31							
pH STANDARDS		7.00	10.00	4.00	7.40		
SUPPLIER/I.D.							
LOT NUMBER							
EXPIRATION DATE							
NOTES:							

ADDENDUM IV (cont.)

EXAMPLES OF LABORATORY EQUIPMENT LOGS

MONTH________________ YEAR__________________			RERIGERATOR I.D. NUMBER______________ LOCATION________________	FREEZER I.D. NUMBER______________ LOCATION________________
DATE	**TIME**	**INITIALS**	**TEMPERATURE (^{O}C.)**	**TEMPERATURE (^{O}C.)**
1				
2				
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6				
7				
8				
9				
10				
11				
12				
13				
14				
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31				
NOTES:				

ADDENDUM V

SUGGESTED STANDARD TEST REPORTING TEMPLATE FOR STUDY WORKBOOK

[1]SOLUBILITY TESTING
Test Chemicals for the *In Vitro* Validation Study

Study No.___________________

Test Chemical_________________ Test Chemical Code__________ CAS #____________

Physical Description_______________________________________ Liquid Density_________

Solubility Determined by__________________________ Date______________

Solvent	Amount of Test Chemical	Volume Added	Total Volume	pH and medium color	Vortex (V) Sonication (S) Heating-37^{o}C (H)	Comments
Treatment Medium (3T3 NRU)		0.1ml				
		0.5ml				
		1.0ml				
Routine Culture Medium (NHK NRU)		0.1ml				
		0.5ml				
		1.0ml				
DMSO		0.1ml				
Ethanol		0.1ml				

Reference Color of Treatment Medium_______________________

Reference Color of Routine Culture Medium___________________

Balance I.D.______________

Treatment Medium and Routine Culture Medium: minimum concentration of 100mg/ml.
DMSO and Ethanol: minimum concentration of 1000mg/ml.

[1] Adaptation of Institute of In Vitro Sciences (IIVS) form – 350 [2/2002]

ADDENDUM VI

GANTT CHART OF STUDY TIMELINES AND DELIVERABLES

In Vitro Cytotoxicity Validation Study			MARCH 2002	APRIL 2002	MAY 2002	JUNE 2002	JULY 2002	AUGUST 2002	SEPTEMBER 2002	OCTOBER 2002	NOVEMBER 2002	DECEMBER 2002	JANUARY 2003	FEBRUARY 2003	MARCH 2003	APRIL 2003	MAY 2003	JUNE 2003	JULY 2003	AUGUST 2003	SEPTEMBER 2003	OCTOBER 2003	NOVEMBER 2003	DECEMBER 2003
TASK	**START**	**FINISH**																						
Statement of Work Issued by NIEHS		3/29/02	29																					
Proposal received		5/10/02			10																			
Contracts Awarded		6/29/02				29																		
Submission of Study Protocol, CVs of Key Personnel, and SOPs		7/12/02					12																	
Phase Ia Positive control	7/29/02	8/26/02					July 29 Aug. 26																	
Phase Ia Draft Report		9/9/02						Sept. 9																
Phase Ia Final Report		11/11/02							Nov. 11															
Phase Ib 3 chemicals	9/26/02	10/29/02							Sept. 26 Oct. 29															
Phase Ib Draft Report		11-11/02								Nov. 11														
Phase Ib Final Report		1/13/03									Jan. 13													
Phase II 9 chemicals	12/2/02	2/10/03											Dec. 2 Feb. 10											
Phase II Draft Report		2/25/03											Feb. 25											
Phase II Final Report		4/28/03												April 28										
Phase III 60 chemicals	3/26/03	12/9/03																	Mar. 26 Dec. 9					
Phase III Draft Report		10/24/03																	Oct. 24					
Phase III Final Report		12/9/03																	Dec. 9					
Biweekly Reports	7/10/02	12/9/03													July 10, 2002 – December 9, 2003									

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